EXTRACTION METHODS OF DIATOMS-A REVIEW

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ABSTRACT:

Detection of diatoms in tissues has been applied as an important sign of drowning since the beginning of 20th century and utility of diatoms for the diagnosis of drownings cases was debated soon after they were first found in lung exudates. Hard bones (sternum and femur) and soft tissues (lungs and liver etc) of drowned bodies are usually sent to the Forensic Science Laboratories for the detection of diatom. Dissolution of these samples is not very difficult but complete extraction of diatoms frustules from these samples needs great care, attention and expertise. While solving drowning cases, a correlation between the diatoms extracted from these tissue samples and the samples obtained from putative drowning medium has to be established for the successful determination of drowning site. Therefore, it needs proper method for extraction of diatoms from tissues and water samples to avoid even minor contamination. In the present study, literature related with various extraction methods of diatoms from postmortem and water samples has been reviewed.

Keywords - Drowning, Diatoms, Bone marrow, Centrifugation

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DISCUSSION

Role of diatoms has always remained significant in solving the drowning cases. In cases, where the cause of death cannot be ascertained by conventional post-mortem examination in those cases presence of diatoms in lungs and other body tissues plays an important role in determining whether the death is due to drowning or not.

Diatoms are unicellular having a cell wall made of silicon dioxide. The glass frustule is composed of two valves, which fit together with the help of a cingulum, or set of girdle bands. There are about 10,000 species and 174 genera of diatoms reported which are having different shapes and sizes varying from 1 to 500µm. Diatoms are traditionally divided into two orders: centric diatoms (Centrales), which are radially symmetric, and pennate diatoms, which are bilaterally symmetric (Pennales) but Round et al. (1990), classified diatoms into three classes: centric diatoms (Coscinodiscophyceae), pennate diatoms without a raphe (Fragilariophyceae), and pennate diatoms with a raphe (Bacillariophyceae). Most diatoms exist singly, although some join to form colonies. They are usually yellowish or brownish, and are found in fresh and salt water, in moist soil, and also on the moist surface of plants.

Analysis of diatoms present in the body tissues like lungs, liver, spleen, blood and bone marrow has been undertaken as supportive evidence in downing cases. The theory behind the 'diatom test' is that when any person drowns, the diatoms present in that water will reach to the lungs and some of them because of their size penetrate into the alveoli. If the heart is still beating, the diatoms that have entered into the blood stream travel around the body and may lodge in distant organs such as the kidneys, brain and bone marrow before death. The presence of diatoms in the internal organs most likely confirms the ante-mortem drowning. If there is any doubt about the drowning site, then water sample from the putative site of drowning can be collected and analysed to determine the similarity of different species of diatoms in the water and the body.

For last 100 years, many scientists have employed various extraction methods individually and in combinations in order to isolate diatoms from water and tissues samples successfully. Among these approaches some of them were found to be superior to others. Nitric acid digestion is a worldwide known

method for the extraction of diatoms. Apart from this some other extraction methods like Ultrasonic radiation, Enzymatic digestion and Physical methods such as simple centrifugation and gradient centrifugation method are also employed for this purpose.

ACID DIGESTION METHOD

This method had been a revolution in the history of diatoms extraction and accepted worldwide. It is characterized by its low cost, easy to perform and gives excellent results. Mueller and Gorgs (1949); Mueller (1952); Naeve (1956); Thomas et al. (1961); Bhaskar (1965); Timperman (1962); Spitz and Schneider (1964); Porawski (1966); Neidhart and Grrendyke (1967); Koseki (1968); Hendey (1973); Nanikawa and Kotoku (1974); Khattab (1975); Udermann and Schuhman (1975); Peabody (1977); Schellmann and Speri (1979); Foged (1983); Calder (1984); Auer and Mottonen (1988); Pachar and Cameron (1992 and 1993); Taylor (1994); Pollanen (1998); Pollanen (1997); Pollanen (1998); Azparren et al. (1998); Hurlimann et al. (2000); Gruspier and Pollanen (2000) and Kazutoshi et al. (2004) are among those renowned workers who favoured this method for dissolving tissues samples.

Extraction of diatoms from tissue samples using acid digestion method:- Thomas et al. (1961) and Timperman (1962) dissolved sternum bone marrow in a Kjeldahl flask containing 50 cc of nitric acid. After half an hour the yellow fluid turned transparent. This solution was allowed to cool and then centrifuged. The residue was put on the slide and examined under a microscope. But Pollanen et al. (1997) made a slight modification in this method by removing bone marrow (50 gm) from femur bone and put into a boiling flask. Approximately, 50 ml. of concentrated nitric acid (analytical grade) was added and marrow-acid suspension was simmered on a hot plate for approximately 48 hours in a fume hood. After cooling suspension at room temperature it was centrifuged (200-300 g/30 min). The residue so obtained was then added in distilled water and again centrifuged. Then final supernatant was discarded and pellet containing nitric acid-resistant material was aspirated using a Pasteur pipette and put on a clean microscopic slide for examination.

Peabody (1977) also extracted diatoms by removing organic matters using acid digestion method. But this time traces of $CaCO_3$ were removed by adding conc. HCI. It was again added with conc. H_2SO_4 and boiled until suspension turned black. Supension was allowed to cool and solid $NaNO_3$ was added to it. Suspension was reheated until colour turned to brown and finally clear (HNO_3 produced oxidized the carbon to CO_2). Resultant suspension was washed thoroughly in diatom free water and re-suspended in acetone for the preparation of permanent slides.

Yange et al. (1999) development a new instrument called ‘can’ it was also based on acid digestion procedure. This instrument overcame the shortcomings of previous methods for the destruction of organic material so that their identification became easier. ‘Can’ consisted of three parts - a can body, an inner cover and an outer cover. For destruction procedure, the organic material was filled with Teflon, which made this instrument as corrosion resistance, heat resistance, pressure resistance, and leak proof etc. Under the fit of strong acid reaction and high temperature action, organic tissues were liquefied for the extraction of diatoms. 3 gm of tissue sample was added with 4 ml of strong nitric acid in this instrument. Can was then placed in a dry box at 102°C for 100 min. Then ‘can’ was cooled and postdigestive liquid was filled in centrifugation tube. After centrifuging with distilled water, residue so obtained was put on slide for further analysis. In Japan many instruments were used for chemical digestion of tissue materials by making modifications as reported by Tomonaga (1954). Samples were digested with fuming nitric acid and sulphuric acid in a water bath (60°C - 180°C) or on a sand tray (80°C-300°C). Solution was centrifuged and added distilled water in it and this method was called ‘Disorganization method’. In a highly modified method, Krstic et al. (2002) treated 2 g of tissue sample with H_2O_2 for 24 hours. After finishing the oxidation process, H_2SO_4 was added in amount necessary to complete the oxidation of organic matter. Saturated solution of potassium permanganate turned this solution violet and oxalic acid again discoloured it. After 48 hours of sedimentation, the probe was leveled to approximately 100 ml and centrifuged at 3000 rpm for 20 min, until the pH became neutral. Permanent diatom slides were prepared using the residue material.

Extraction of diatoms from water samples using acid digestion methods:- Tyagi (1985) collected water samples from various water bodies like lakes, ponds, wells and drains in Delhi. Water samples were treated with conc. HCI acid and supernatant was discarded. Then addition of conc. H_2SO_4 turned it blackish (conc. H_2SO_4 charred present organic material). The supernatant was cooled and added with solid $NaNO_3$. Suspension was reheated until its color turned brown and finally cleared. Distilled water was used for the washing of the resultant suspension of silica diatom cells and residue was re-suspended in acetone. Pollanen (1998) also extracted diatoms from putative water samples using acid digestion method. While Kazutoshi et

al. (2004) collected 200 ml of sea water samples from the surface and bottom and 10 mL of the collected sample was concentrated to 0.2 mL by centrifugation using a tabletop centrifuge at 3,200 rpm for 10 min with a 10 mL centrifugation tub. The concentrated seawater was incubated with fuming nitric acid for 30 min in boiling water. Sample treated with acid was washed twice with pure water and twice with pure ethanol by centrifuging at 3,200 rpm for 10 min. Residue was heated and dried on glass slide and diatoms were examined with an optical microscope at a magnification of 400-fold. Bhatt et al. (2001), using a sharp-edged knife colleted epilithic diatom samples by scraping the rocks and boulder surfaces (3 mm sq). The epilithic diatom samples obtained were cleaned with nitric acid and potassium dichromate. The samples were centrifuged at 10,000 rpm for 10-30 min and the supernatant was discarded. The pellet was washed twice with Isopropanol, followed by a single wash with Xylene. The pellet was suspended in distilled water and permanent mounts were prepared in Canada balsam.

SOLUENE-350 METHOD

Sidari et al. (1999) used Soluene-350 method for the extraction of diatoms from fresh water and seawater samples. Samples were taken from lungs, liver and kidney of three drowned subjects. Seawater samples were collected with a phytoplankton-net while scraping the stones and shaking the macro algae collected freshwater samples. At the time of collection water samples were fixed with formalin. 30 ml. of both fresh and seawater samples were washed thrice with distilled water and at every wash, samples were centrifuged at 3000 rpm for 5 min and the supernatant was discarded every time. Pellet was suspended in 8 volumes of Soluene-350. The solutions were then incubated at 50 °C for 2 hours and subsequently at room temperature overnight. Following centrifugations at 3000 rpm for 60 minute, samples were analyzed using the light microscope. For all tissue samples negative results were obtained, but as far a water samples were concerned Soulene-350 reacted positively. Treatment was excessively destructive for seawater diatoms, probably because their frustules were less silicized and have less resistance as compare to the fresh water diatoms. Earlier in 1980, Fukui et al. had employed this method for destruction of tissue samples. Soluene-350 (Packard Instruments), NCS (Amrsham/Searle Corp.) and Protosol (New England Nuclear) were used to solubilize the liver and lung samples of rabbits. Ultrasonic irradiation was used in place of strong acids and three procedures were used for the preparation of samples; (I) 1 gm of tissue sample was cut into small pieces using scissor following the addition of 20 ml of solubilizer, (II) same quantity of tissue sample was crushed in mortar after being cut, and was suspended in toluene. The solubilizer was then added to the suspension and (III) again same quantity of tissue sample was homogenized in 9 ml of distilled water. The homogenate was centrifuged at 15,000 rpm for 10 min and 5 ml of the precipitate was added with solubilizer.

For examining the practicability of the method, tissue sample was homogenized with water containing diatoms. Centrifugation and digestion procedures were same as mentioned above. After complete digestion, the solution was centrifuged at 3000 rpm for 5 min. approximately 1 ml of the precipitate was re-suspended, 1 drop of which was examined microscopically for the presence of diatoms. Ultrasonic irradiation for 300 min resulted in almost complete digestion of the tissue. However, some tissue fragments still remained even after 300 min, where radiations (ultrasound) could not be subjected properly. Among the solubilizers, Soluene was reported as best solubilizer for tissue digestion. Slight destruction of diatoms due to ultrasonic irradiation was observed with both direct and indirect irradiation but there was not much difficulty in recognizing diatoms.

ENZYMATIC METHOD

The reliability and applicability of qualitative and quantitative diatom analysis was evaluated by Ludes et al. in 1994 using an enzymatic digestion method for the diagnosis of putrefied drowned bodies. Tissue samples were treated with both chemical and enzymatic methods using concentrated nitric acid and Proteinase-K respectively. Enzymatic method was considered more convenient in terms of rapidity, safety and environmental protection than chemical test. In 1996 and 1999 Ludes et al., made use of hydrogen peroxide (130 vol. %) at 80oC for 12 hours for the treatment of the water samples. The solution was cooled at room temperature and second centrifugation was set at 2500 rpm for 15 min. After discarding the supernatant, finally centrifuged with distilled water (3000 rpm) produced a pellet containing diatoms. After removing the supernatant, the sediment was air dried and mounted in Naphrax. Diatoms were examined under light microscope (Zeiss, Germany) equipped with an oil immersion objective (1000X).

For tissue samples, 10 g of each of the organs (peripheral lung tissue, kidney, liver, brain and femur bone marrow) was minced with scissors. Sample was rinsed and mixed with 500 ml of 10 mg/ml Proteinase-K and 100 ml of 0.01 M Tris-HCl buffer (pH 7.5) containing 2% SDS. The mixture was incubated at 50 °C overnight, 500 ml Proteinase-K (Boehringer Mannheim, Germany) was added and the solution was diluted with 100 ml.

of distilled water and centrifuged at 3000 rpm for 15 min, the upper layer was then removed. The sediment (100 ml/slide) was transferred on a cover glass, mounted in Naphrax and examined under the light microscope. Azparren et al. (1998); Kobayashi et al. (1993); Taylor (1994) and Quantin et al. (1994) also suggested use of Proteinase-K for the extraction of diatoms from the tissue samples of drowned bodies.

MEMBRANE FILTER METHOD

This method was considered very useful to solve problems which disturb microscopic observations i.e. destruction and loss of diatoms and appearance of inorganic crystals. In 1987, Funayama et al. first time introduced this method for the extraction of diatoms from blood samples, and in the same year they again used this method for the extraction of diatoms from the blood and tissue material. In this method, filtration was done using a membrane composed of nitrocellulose type with a pore size of 5 µm. 5 ml. of blood and 10 ml. of 5% Sodium Dodecyl Sulfate (SDS) were mixed and stirred slowly. The resulting hemolysate was filtered through a membrane filter of 47 mm in diameter. When blood was putrefied or extremely clotted, pores of the filter got choked. In these cases, the filter had to be changed with another one. After filtration, the filter(s) were digested with about 10 ml. of fuming nitric acid for about 10 min. After cooling, this solution was diluted 10-20 times with distilled water. This dilution was filtered through another membrane filter measuring 25 mm. in diameter, and this filter was dried and observed under microscope. While treating tissue samples, the residue obtained by nitric acid digestion was diluted with 150-20 ml. of distilled water and filtered through a membrane filter of 47 mm. in diameter. Fatty material remained on filter was digested completely with isopropyl and petroleum ether alternately.

Mottonen and Ravanko (1971) and Funayama et al. (1987) adopted this method for examining diatoms and pollen grains in arterial blood of drowned victims. Membrane filter method was a method of choice far the extraction of diatoms from the blood. While in another experiment Rammer and Gerdin (1976) collected blood samples from right and left side of the heart of drowned persons. The samples of cerebrospinal fluid and vitreous humor were also collected by aspiration. These fluids were centrifuged and the supernatant frozen after thawing. Karkola and Neittaanmaki (1981) preserved blood samples by adding them with Heparin and antibiotic. To each 5 ml. of sample, equal volume of distilled water was added and followed by centrifugation at 4000 rpm for 10 min. About 10 ml. of water was added to the residue and centrifugation at 2000 rpm was carried out for 10 min. This procedure was repeated. The fourth sequence of this gradual haemolysis by dilution was made with Hank's solution in order to remove the debris of RBCs. Smear was made from the residue on slide and stained by using Giemsa method. Diatoms were found only in a few drowning cases.

Extraction of diatoms from 'air' samples:- Spitz and Schneider (1964) conducted an experiment, where diatoms were extracted from air samples using air 'filtration bands'. Over a length of 90 cm around 500 m3 of air was filtered daily through this method. Bands were incinerated, and remaining ashes were suspended in a 10% solution of hydrochloric acid. A three days survey in April and another three days survey in June 1961 revealed 662 and 1546 diatoms respectively. Examination of new bands, prepared for microscopy with utmost precautionary measures to avoid contamination, showed no diatoms over a length of 96 cm. In another effort Neidhart and Grrendyke (1967) extracted diatoms with filtration technique. Air sediment collected over 30 days period in 1 feet square sediment traps was charred to destroy organic material present and the remains were examined microscopically. Air sediment was also investigated by filtering outside air through a millipore filter apparatus (5.0 µ pore size) for 2 hours. Trapped debris was observed under microscopic using oil immersion.

COLLOIDAL SILICA GRADIENT CENTRIFUGATION METHOD

Terazawa and Takatori (1980) homogenized 0.5 g of lung and 1.0 g of liver samples with distilled water in a Potter-Elvehjem homogenizer in cooling conditions. Similarly 5 g of human lung sample was also homogenized with same procedure but in this case saline was used in place of distilled water. 9 ml of Percoll (colloidal silica particles, Pharmacia fine chemicals) and 2.0 ml of the homogenate were mixed well with a Vortex mixer and filled in a translucent, soft plastic centrifuged tube (12 ml) made of polyallomer. For the Percoll mixture centrifugation was set at 17,000 rpm (35,000 g) for 60 min at 12°C using a refrigerated centrifuge (Hitachi model 20-PR). Upper layer containing cell debris was discarded and residual Percoll portion was added with two volumes of distilled water. Mixture was well stirred and again centrifuged at 3000 rpm for 5 min and the supernatant was removed. Washing procedure was repeated twice and maximum of the final washing solution was removed. An aliquot of the sediment suspension was put on slide glass with help of a micropipette and microscopy completed further analysis.

DRY ASH METHOD

In this method 5 g of bone marrow or any other tissue sample was mixed in nitric acid and burnt in a muffle furnace for the extraction of diatoms. It was an ideal method for the good recovery of diatoms from tissue samples, since it was rapid method and only small amounts of acids were required (Peabody, 1980). During survey of literature for the treatment of water samples, no significant information regarding this method could be found.

Preparation of Diatom Slides for Scanning Electron Microscopic (sem) Analysis

Torre et al. (1983), and Torre and Varetto (1985) described the treatments of tissue sample for the extraction and preparation of diatoms slides. 0.1M-phosphate buffer solution (pH 7.4) was used for the washing tissue samples and this solution was fixed in 2.5% glutaraldehyde for about 4 hours. After second washing with same buffer, samples were dehydrated using grade alcohol series and then dipped in amyl acetate. Completely dried specimens were finally gold coated for analysis under Cambridge 250 Stereoscan. Lunetta et al. (1998) slightly modified the extraction method, where tissue samples were immersed in 3% glutaraldehyde for 1 hr. A tissue sample measuring 1X1 mm. was cut and prefixed in 3% glutaraldehyde in 0.1% phosphate buffer. The sample was first dehydrated in a grade ethanol series and dried in Blazer CPD 020 critical point dryer. At last sample was mounted on stub and coated with gold in a Jeol Coat-Ion sputter JFC 1100 for 10 min. All samples were observed systematically with a Jeol JSEM-820 SEM operating at 10-40 kV. Samples for Transmission Electron Microscope were prefixed in a phosphate buffer 1% at 6°C for 3 hours, dehydrated in a graded ethanol series, infiltrated with propylene oxide and embedded in Ladd's epon LX-112. From both edges of all blocks (n=250) 20 semiserial (Int.val 20µm) semi fine sections (0.5µm) were prepared and stained with toluidine blue for light microscopic (LM) analysis. Pachar and Cameron (1992 and 1993) used SEM for the qualitative analysis of diatoms in drowning cases. Block of approximately 100 gm of the organ was cut by avoiding contamination, dropped into new Kjeldahl flasks, and boiled in twice the volume of concentrated analytic nitric acid under a fume hood. The fluid obtained was allowed to cool at room temperature; it was centrifuged at 2500 rpm for 25 min, and the supernatant acid was discarded and replaced with diatom free distilled water. This process was repeated thrice at 3000 rpm to produce a small button of deposit. After removing the supernatant, sediment was air dried on a metallic stub for SEM, and then coated with a conductive layer. Fatty sediments from bone marrow, brain and kidney were also transferred into the SEM stubs for air-drying on a hot plate at 60°C. Sediment of the water sample was also air dried, coated, and examined with same procedure.

CONCLUSION

Treatment with 'Acid digestion' method is used in the majority of the trials. This method is easy and a better means to remove organic materials. Besides having many merits this method also has some demerits like substantial amounts of acids are required to digest 100 gm of tissue sample. A high-speed centrifugation is required to remove traces of acids, makes this method lengthy. Sometimes, strong corrosive acids may affect delicate types of diatoms. Chemical digestion of fatty materials may be time consuming and sometime completely not removable. Chances are there that diatoms may be lost in repeated washings and centrifugations. As Kjeldahl flasks and reflux apparatus are costly therefore they are reused after washing, so acid digestion is prone to contamination from the reagents and glass wares also. It is possible that washing of this type of apparatus after several operations, when the glass surface is etched, can never reach the standards required. No doubt, acid digestion method is effective for detecting diatoms, but necessarily not safe. Strong acids are heated and inhaling of eliminating harmful gases; such as nitrogen oxide and sulphur dioxide can cause heath hazardous. Detection of planktons other than diatoms by this method is not possible.

Using 'Hydrogen peroxide' as a means of oxidizing the organic material is not advisable because complete washing off peroxide traces from the samples is difficult due to the bubbling of the solution but in acid digestion method nitric acid can be washed off easily. 'Enzymatic digestion' method is simple, safe and effective for detection of both phytoplankton including diatoms and zooplanktons, which is not possible in the acid digestion method. Therefore this method is more advantageous than acid digestion methods, in diagnosing drowning cases. Using subtlisin, this is an effective and rapid method of destroying tissue materials and Tris buffer can be easily cleaned by centrifugation. But method is bit costly than other traditional methods.

In 'Membrane filter' method many crystals and other particles from the blood samples are absorbed on this

membrane filter, which can create a problem in detection of diatoms. In the beginning this method was found unfit for the extraction of diatoms from the degenerate and putrefied blood samples but use of nitric acid made this method applicable for both blood and tissue samples. 'Dry Ash method' is less practicable however if this method is to be applied then 100 gm of tissue sample have to be divided into several aliquots. Ash of organic material seems to be a poor method, because it is suspected that it can badly affect some diatoms species. 'Colloidal silica gradient' is successful in detecting some species of planktons other than diatoms, however during homogenization large planktons are destroyed and some contamination is supposed to occur in case of decomposed tissue. Despite having many limitations, acid digestion method is still better and very frequently used for the extraction of diatoms from water and tissue samples.

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